Fluorometric Determination of Thiamine Vitamers in Chicken

A comparison was made of the direct determination of thiamine in acidified heated chicken extracts, by either flow injection or chromatographic determination, with the standard method (acid and enzyme digestion, adsorption, and elution, followed by the fluorometric determination of thiochrome extracted by isobutanol from $K_3Fe(CN)_6$-treated eluates). Liquid chromatography of extracts, followed by oxidation of thiamine vitamers to thiochromes, showed 1 light scatter emission peak and 2 thiochromes, the latter corresponding to thiamine and thiamine monophosphate. Both forms were determined quantitatively by flow injection determination, the lower detection limit of which was about 60 femtomol. The determination was linear from 0.1 ng to 10 μg thiamine/mL, and the pooled coefficient of variation was 4%. The determination of thiamine in chicken extracts provides a nondestructive method for determining thiamine and its phosphate esters, either *in toto* by flow injection determination or as individual components by chromatography.

During the course of the study of the effects of ionizing radiation on thiamine in chicken, we had occasion to determine the concentrations of the various thiamine vitamers: thiamine (Thmn), thiamine monophosphate (TMP), and thiamine diphosphate (TDP, cocarboxylase). In the standard method of thiamine determination (1, 2), the last 2 vitamers are dephosphorylated by an enzyme digestion step because, for the measurement of the thiochrome formed from the oxidation of thiamine, the thiochrome is extracted into isobutanol in which the thiochrome phosphate esters are not soluble. This method does not distinguish the vitamers from each other. Liquid chromatography obviates the need for cleanup (2–16) and determines the phosphate esters individually, but most of the methods studied (3–12) involve precolumn oxidation to thiochrome, which destroys the vitamers before they are separated.

We determined the thiamine vitamers directly in an aqueous chicken extract, but the solutions were too turbid for fluorescence measurements because of Rayleigh and Raman scattering. Acidification and heating (equivalent to the AOAC rapid method, **953.17** (1)) yielded clear solutions suitable for measuring thiochrome fluorescence, but we consistently obtained higher values for the thiamine content (ca 1.60 μg thiamine/g meat) than those reported in the literature (ca 0.4–0.8 μg/g) (3, 8, 17–20). Therefore, we examined the thiamine content of chicken breast extracts at each step of the sample preparation to ascertain if the lower values obtained from the standard method were due to losses in 1 or more of the steps. We also chromatographed the extracts on different resins to ascertain if we were measuring an artifact. We extended the study to the determination of the thiamine vitamers *in toto* by flow injection determination.

Experimental

Reagents

All thiamine vitamers were obtained from Sigma Chemical Co., St. Louis, MO 63178: thiamine hydrochloride (T4625, lots 125F-0250 and 94F-0334), thiamine monophosphate chloride (T8637, lot 93F-00391), and cocarboxylase (C-8754, lots 106F-0182 and 110G-2420). For accurate determination of the fluorescence intensity, the vitamers were dried over silica gel at 84°C. By chromatography, thiamine was found to be a single component, but both thiamine monophosphate and cocarboxylase contained varying amounts of thiamine, probably due to decomposition of the phosphate esters during storage. All other chemicals were reagent grade and all solutions were prepared in deionized/distilled water. As necessary, eluting solutions were filtered through 45 μm Supor-450 membrane filters and either sonically degassed or purged with helium.

Equipment

Samples were injected into the buffer stream by either an ISIS autoinjector or a Rheodyne 7125 sample injector (Rheodyne, Inc., Cotati, CA 94931), both with 200 μL loops. Two fluorescence detectors were used: a Waters 420 fluorescence detector with a F4T5/BL lamp (Waters Chromatography, Milford, MA 01757), 365 nm excitation filter, and a 425 cut-off emission filter, and a MacPherson FL-750 photofluorometer, $\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 460 nm, with either a 400 or a 440 nm cutoff filter. MPF-44E spectrophotofluorometer was used to measure the fluorescence spectra (Perkin-Elmer Corp.,

Norwalk, CT 06859). For scanning the excitation spectra, the emission wavelength was set at 435 nm and the excitation spectrum was scanned from 325 to 400 nm. For the emission spectra, the excitation wavelength was set at 435 nm and the excitation spectrum was scanned from 380 to 500 nm.

Sample Preparation

Figure 1 is a flow diagram of sample preparation by the standard method. Asterisks indicate the steps after which samples are removed for thiamine determination. The double asterisk indicates the point at which samples are removed for the rapid method.

Slurry

Fresh chicken breasts were obtained from a wholesale dealer, usually 1 day after slaughter, although some breasts were obtained the first day. The skin and excess fat were removed, and the meat was separated from the bone and sliced into 1/4–1/2 in. cubes. For homogeneity and ease of handling, 80 g of the meat was blended 15 s with 160 mL water under nitrogen in a glove bag. (We felt the precaution of blending under nitrogen was advisable because the blending process introduces a large amount of gas into the liquid.) The resulting slurry is a highly homogeneous material for the study of sample preparation; it was easily transferred quantitatively by aspiration into a 50 mL irrigation syringe and transferred by weight into appropriate containers.

Sample Sets

For all runs, sample sets consisted of a water blank, a thiamine vitamer standard, a chicken extract, and a chicken extract spiked with the standard.

Sample Preparation, Standard Method

To determine the thiamine concentration at each step of the standard method, the steps had to be modified slightly. For the HCl extracts, 8 mL 1N HCl was added to 90 g slurry to lower the pH to 1.5, and the mixture was stirred vigorously. The slurry was then drawn up into an irrigation syringe and 16.3 g was transferred into 50 mL Erlenmeyer flasks; 2 sample sets were prepared. Then, 15 g water was added to the zero concentration and the standard flasks. For the standard and spiked samples, 0.5 mL of a stock solution of 10 μg thiamine/mL was added to the appropriate tubes. Next, 1.5 mL 1N HCl was added to all flasks in both sets, and each flask was diluted to ca 35 mL. The flasks were stoppered with rubber stoppers covered with Saran wrap and heated 30 min in a boiling water bath. The flasks were cooled and the contents were adjusted to pH 4.5–4.7; one sample set was diluted to volume to serve as the acid digestion sample. Then, 2.5 mL 5% α-amylase was added to each of the flasks in the second sample set, and the set was incubated overnight at 37.5°C. We found that these time and temperature conditions were necessary for complete conversion of TMP to thiamine by the α-amylase preparation we used (cf 2, 7). The samples in this set were transferred to 100 mL volumetric flasks and diluted to volume. A 25 mL aliquot of each sample was placed on a Bio-Rex column prepared as described below, washed with 20 mL 70°C water, and eluted with 70°C acid–KCl into 25 mL volumetric flasks. Next, 2.5 mL of each column eluant was placed in a centrifuge tube (capped type), 2.5 mL 0.04% $K_3Fe(CN)_6$ and 7.5 mL isobutanol were added to each tube, and the tubes were capped and shaken lightly for 2 min. This last operation was performed in subdued light coming from the windows 30 ft away (thiochrome is sensitive to light), and in a hood (isobutanol is mutagenic). After the tubes were allowed to stand 1 min, the isobutanol supernatant cleared and was suitable for thiochrome determination in a fluorometer.

A total of 5 sample sets was obtained: (*1*) TCA/heat treated, (*2*) HCl/heat treated, (*3*) HCl/heat treated after enzyme digestion, (*4*) HCl/heat treated after adsorption/elution on Bio-Rex 70, and (*5*) HCl/heat treated after thiochrome formation/isobutanol extraction.

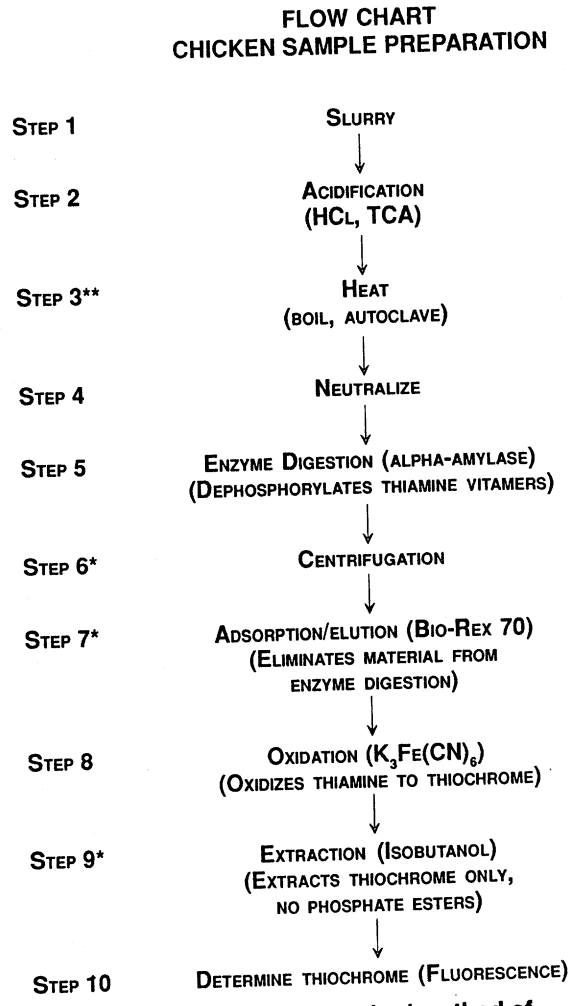

Figure 1. Flow chart for the standard method of thiamine determination. Asterisks indicate the step after which samples were taken for thiamine.

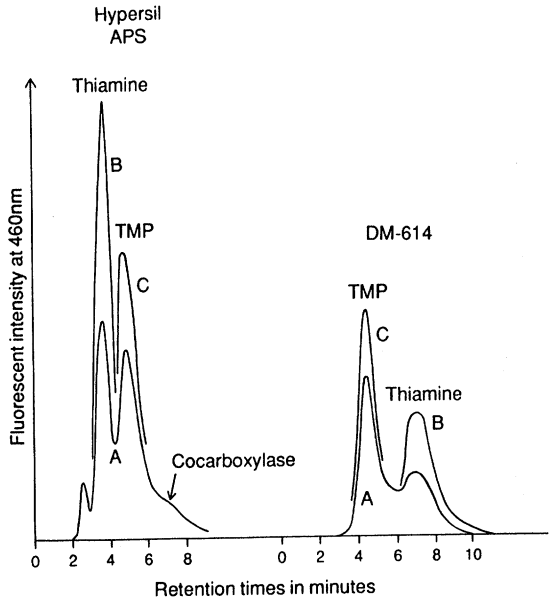

Figure 2. Chromatograms of chicken extracts, with and without spiking with thiamine and thiamine monophosphate, on Hypersil APS and DM-614. Curves A, chicken extract. Curves B, same with thiamine spike. Curves C, same with thiamine monophosphate spike.

Bio-Rex 70 Preparation and Use

Bio-Rex 70 resin, 50–100 mesh, Control No. 33772, was used to purify thiamine in chicken extracts. About 20 g resin was washed with distilled water, and the fines were decanted. The resin was then washed with four 250 mL portions 1N HCl, and the resin was allowed to stand for a short period of time in the acid. The resin was washed until the washings were neutral to pH test papers. The columns consisted of a 50 mL tube sealed to a column 13 cm long by 6 mm id, with a Luer tip sealed to the end. A 3-way valve was placed on the Luer tip, and a 22 g needle (square tip) was attached to the valve. The column was plugged with polypropylene wool and filled to 10 cm with the washed resin. With the mesh used and the 22 g needle, the flow rate was a little over 1 mL/min.

Sample Preparation, Rapid Method

Samples were acidified with either trichloroacetic acid (TCA) (15, 21) or HCl (1, 2). For the TCA extracts, 9 g slurry was weighed into centrifuge tubes (polyallomer, capped type) and 18 mL 2% (w/v) trichloroacetic acid was pipetted into the tube. For the zero thiamine concentration and standard samples, 9 g water was weighed into tubes. For the standard and spiked chicken extracts, 0.3 mL 10 μg/mL standard thiamine was added to the appropriate tubes. The tubes were capped tightly, shaken vigorously, and heated 30 min in a boiling water bath. After cooling, the tubes were shaken again, and centrifuged 15 min at 20 000 × *g* and 5°C.

Columns

For chromatographic separation of the thiamine vitamers, we used either Shodex's DM-614 (equivalent to a C3-4 reversed-phase) (22) or Hypersil APS (anion exchange) columns (Chrompak B.V., Middelburg, The Netherlands). The eluting solution for the DM-614 and Hypersil APS was 0.05M citrate buffer, pH 4.5. The columns were run at room temperature; any variations in retention times were compensated by including standards in all runs.

Determination of Thiamine

Thiamine was determined in either the standard or rapid method extracts by flow injection determination (FID) or after chromatographic separation (CD), except for the isobutanol-extracted thiochrome, for which the fluorescence was measured in a 1 cm sealed cuvette. After sample injection into the buffer stream, either with or without a column, a solution of 0.04% $K_3Fe(CN)_6$ in 2% NaOH was mixed into the stream at the same flow rate as the buffer stream, and allowed to flow through a reaction coil 160 × 0.060 cm id at room temperature. Although this length of coil allows only 0.5 min of reaction time, we found, as Cooper and Matsuda (13) observed, that the oxidation of thiamine by ferricyanide is exceedingly rapid. The thiochrome produced by oxidation of thiamine by ferricyanide was determined fluorometrically in a 12 μL flow cell.

Results

Before we initiated the major study, some preliminary studies were performed. We tried the direct addition of ferricyanide to the chicken extracts, but the results were erratic and generally low. The indicated heating step was found necessary to obtain clear extracts and eliminate reduced yields for both the trichloroacetic and hydrochloric acid extracts. The usual concentration of alkali for the oxidation step is 15% (w/v), but successive dilutions showed no difference in the measured thiochrome until about 1% (w/v) NaOH. The desired alkaline conditions of about pH 13 were produced by choosing 2% (w/v). Concentrations of ferricyanide above 0.1% resulted in decreased yields of thiochrome, probably through further oxidation of the thiochrome formed (23). The only 2 thiamine vitamers found in any great quantity in the chicken breasts we studied were thiamine and thiamine monophosphate; cocarboxylase was present in only very low quantities. The exception to this observation occurred when the chicken was slaughtered the same day, in which case the cocarboxylase content was distinctly greater. Because the first 2 compounds constituted the bulk of the vitamin present, we focused our attention on them.

Thiamine and Thiamine Monophosphate

These vitamers were identified in the column effluents by their retention time on DM-614 and Hypersil APS resins and by their fluorescent spectra. Curves "A" in Figure 2 represent the chicken extracts on the 2 resins; peaks "B," the changes in the curves when the chicken extracts were spiked with thia-

Table 1. Retention times in minutes

Compound	Hypersil APS	Shodex DM-614
Thiamine	3.8	7.1
Thiamine monophosphate	4.8	4.5
Cocarboxylase	7.0	4.5
Light-scattering material	2.5	8.2

mine; peaks "C," TMP–spiked chicken extracts. The thiamine and thiamine monophosphate peaks had the same retention times as the standards (Table 1), and the fluorescence excitation and emission spectra of the thiochromes were identical to the spectra of the thiochrome standards (Figure 3). The peak identified as TMP disappeared after the digestion by crude α-amylase, showing it to be the phosphate ester. The low shoulder below the cocarboxylase arrow had approximately the same retention time as the cocarboxylase standard, but, as shown, was usually present in only very small quantities. A sharp spike is shown in the Hypersil APS column effluent preceding the thiamine peak, but it had neither an excitation nor an emission spectrum; that is, the peak was a scatter peak due to soluble compounds in the extracts. This peak was the only peak present in the alkali blanks, where it was as high as in the ferricyanide-treated effluents. In the effluents from DM-614 columns, this peak appeared in the alkali blanks and came off the column shortly after thiamine. This peak was highly variable from preparation to preparation, as expected, and it was not always observed.

Trichloroacetic Acid Extracts

The TCA/heated extracts gave uniformly clear solutions, with insoluble precipitates that packed well upon centrifugation. The thiochrome spectra of the chicken extracts chromatographed on DM-614 showed the presence of both thiamine and TMP, with retention times of 7.1 and 4.5 min, respectively (Table 1). There was no indication of cleavage of the phosphate esters. The results from the flow injection determination of thiamine in 12 chicken breasts are shown in Table 2. The first 3 values are not significantly different from each other, but after the adsorption/elution step, the determined concentration of thiamine was about half the initial values, which was a significant difference ($P < 0.05$). The precision of the flow injection determination is shown in Table 3, which summarizes the coefficient of variation for the various steps and sample variation. The first 3 rows are for the determination step in standards and chicken slurries. The next 3 rows show the variation due to the sample preparation procedure, the variation between chicken breasts, and the variation from chicken to chicken. The average value for thiamine in the TCA extracts was highest of all the reliable measurements (excluding enzyme-digested samples) and showed the lowest pooled coefficient of variation (Table 2).

Hydrochloric Acid Extraction

The HCl/heated solutions were not always clear when adjusted to pH 4.0–4.3, the pH used for the enzyme digestion

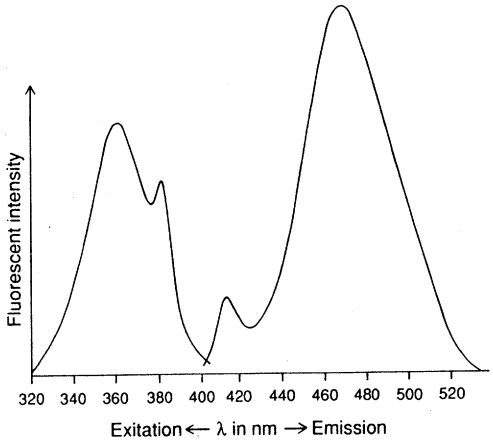

Figure 3. Fluorescent spectra of thiochrome and thiochrome monophosphate, both standards, isolated from chicken extracts by liquid chromatography. All had the same spectra.

(1, 2). This introduced a very high background in the FID or CD separation determinations, and the turbid solutions tended to foul the Bio-Rex 70 columns. Upon investigation, it was found that if the pH of the solutions were brought to pH 4.6–4.8 before centrifugation, clear solutions were obtained (24). The coefficients of variation were quite high: 30.6% for the between-chicken variation and 17.4% for the breast pairs. Of the 2 acids, TCA was preferred over HCl because it produced a clear solution in weak acid solutions, whereas it was necessary to raise the pH of the HCl solutions to a value where thiamine oxidation was a factor.

λ-Amylase Digestion

The enzyme-digested solutions were yellowish, but clear. The chicken samples were generally lighter than the thiamine standards or water solutions, and the spiked chicken samples lighter still, but the differences were not reflected in the fluorescent spectra. That is, the colored compounds did not fluoresce. There was, however, a very large amount of light scattering material, reflected in very high alkali blanks in the FID determination. Chromatography of the enzyme-digested extracts on DM-614 showed that the addition of the enzyme introduced an emission peak that had the same retention time as TMP, but was lower in the thiochrome solutions than in the alkali blanks. That is, the oxidation reduced the fluorescence. When the chicken and spiked chicken extracts were corrected for this difference, no TMP was found in the extracts, as expected after enzyme digestion. The amount of thiamine increased slightly, identifying the peak as TMP, but the increase was not always commensurate with the loss of TMP. When chicken extracts were spiked with TMP, the enzyme digestion eliminated the TMP peak, but there was very little increase in the thiamine peak (Table 4).

Table 2. Determination of thiamine during sample preparation (results of 12 preparations) in μg thiamine/g chicken[a]

	TCA/heat	HCl/heat	Enzyme digestion	Bio-Rex 70 effluent	Thiochrome/ isobutanol
Av.	1.81	1.64	2.26	1.08	0.95
s	0.20	0.40	1.44	1.18	0.30

[a] Values for TCA/heat, HCl/heat, and enzyme digestion are not significantly different; values for Bio-Rex 70 effluent and thiochrome/isobutanol are not significantly different.

After Adsorption/Elution on Bio-Rex 70

The values were uniformly low, averaging 1.21 μg thiamine/mL, about 2/3 of the values were obtained by direct measurement. There was no background fluorescence and only 1 thiamine peak by chromatography.

Thiochrome Formation/Isobutanol Extraction

This is the final step in the usual method of thiamine determination, and it is the principal reason for the enzyme digestion and adsorption/elution steps. Because it is a hand operation, it is inherently less reliable than FID. The coefficient of variation rose to 29%. The average value was 0.95 μg thiamine/mL, which is about the value usually reported for thiamine in chicken but is half the value determined in the acid/heated samples.

Spike Recovery

Spike recovery was the best for the TCA extracts, averaging 99.7% of the standard in preparations 1–12. In a separate experiment, standard solutions of thiamine ranging from 1 to 5 μg/g of chicken were added to portions of a slurry, which was then processed and the thiamine determined. The coefficient of regression was 85.3 units/μg spike/mL, compared to a value of 84.5 units/μg thiamine/mL for the standard. That is, the spike fluorescence was quantitatively the same as that of the standard.

Table 3. Precision of flow injection determination

	Coefficient of variation, %	
Source of variation	TCA	HCl
Thiamine determination		
Standards, $n = 25$		
400 nm cut-off filter	2.10	1.91
440 nm cut-off filter	1.36	0.00
1 slurry, 5 preps., $n = 15$	1.52	0.37
Sample preparation		
1 slurry, 5 preps., $n = 5$	3.37	1.50
Breast pairs, $n = 6$	6.7	17.4
Chicken variation, $n = 12$	15.7	30.6

Attributes of the Method

Specificity

A determinative method is required to be specific, accurate, precise, linear, sensitive, reproducible, repeatable, and rugged. Both the CD and FID measurements were specific for thiamine and/or its vitamers by criteria of retention time and identity of the fluorescent spectra of the standards with those of the vitamers isolated from chicken. As shown by chromatography, the measured emission of the alkali blanks was due to a single light-scatter peak, the magnitude of which was the same in both the alkali blanks and the ferricyanide-treated samples. The sample peaks in FID were, therefore, specific for thiamine and its esters after subtraction of the scatter peak of the alkali blanks.

Linearity and Sensitivity

Linearity was tested in standard solutions and spiked chicken extracts. The determination of thiamine in water or buffer using the MacPherson FL-750 was linear over a range of 0.1 ng thiamine/mL (60 femtomol) to 10 μg/mL. Figure 4 is

Table 4. Thiamine monophosphate loss during enzyme digestion and adsorption/elution

	Thiamine, μg/mL					
	Thiamine		TMP		Total	
Source	Run 16	Run 17	Run 16	Run 17	Run 16	Run 17
TCA extract						
standard (TMP)—	0.56	0.00	0.43	1.00	0.99	1.00
chicken	1.68	2.40	0.54	0.00	2.22	2.40
spike (TMP)	1.88	2.40	1.60	1.00	3.48	3.64
Bio-Rex 70 effluent						
standard	1.00	1.00	0.00	0.00	1.00	1.00
chicken	0.93	0.96	0.00	0.00	0.93	0.96
spike	1.20	1.37	0.00	0.00	1.20	1.38

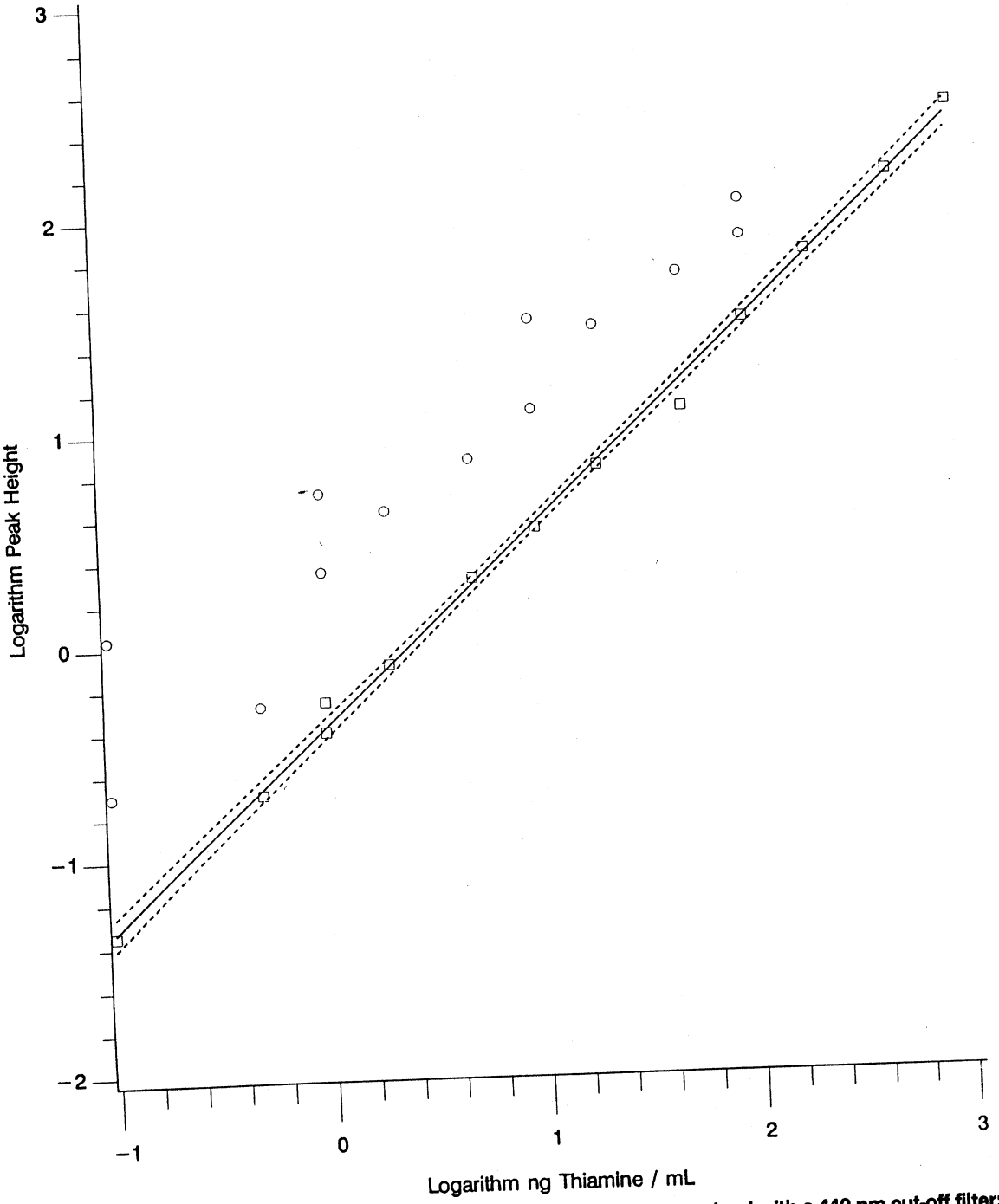

Figure 4. Expression of method sensitivity: square symbols, fluorescence determined with a 440 nm cut-off filter; 95% confidence limits shown are for these data only. Circular symbols, fluorescence determined with a 400 nm cut-off filter in the emission light beam. The latter show the effect of Rayleigh and Raman light scattering at low concentrations of thiamine.

a plot of the lower portion of the curve and shows the effect of using different wavelength cut-off filters. The data represented by the square symbols and the circular symbols were determined by using a 440 nm cut-off filter and a 400 nm filter, respectively, the latter showing the effect of reducing Rayleigh and Raman scattering. Such scattering becomes an important factor at the very low levels of thiochrome fluorescence from the thiamine in chicken. Sensitivity values of 7 and 30 femtomol were reported by Brunnekreeft et al. (6) and Kimura and Itokawa (15) for precolumn and postcolumn oxidation to thiochrome, respectively, compared with our value of 60 femtomol. However, as the sensitivity is equivalent to less than 0.1% of that normally found in the determination of thiamine in chicken, we did not pursue the matter further.

Precision

The results of a quintuplicate preparation/triplicate determination experiment are given in Table 3. The coefficients of variation for the determination of thiamine in a single chicken slurry using 5 preparation samples were 0.37% for the HCl extracts and 1.52% for the TCA extracts. The difference between the HCl and TCA values represents some unknown and erratic stability factor, in either the oxidation process or the output of the fluorometer. At certain times of the day, we did observe great instabilities in the fluorometers, which may have been due to voltage fluctuations.

Accuracy

Confirming our previous observations, the results of the direct addition of ferricyanide to the chicken extracts yielded higher values for the thiamine content of chicken meat than after the adsorption/elution and thiochrome/extraction steps. In Table 2, the first 3 values are not significantly different from each other but are significantly different ($P < 0.01$) from the extracts after adsorption/elution. Because the values after the adsorption/elution step are about the same as reported in the literature (10.8 and 0.95 μg thiamine/g chicken), the modifications made in the method for purposes of the study did not alter the essential character of the procedure.

Repeatability, Reproducibility, and Ruggedness

Repeatability is difficult to determine in the case of thiamine because the vitamin is unstable and varies from chicken to chicken; therefore, a standard concentration sample is impossible to establish. Because the experiment involving the 12 samples was performed over a period of 4 weeks, the measured precision is partly a repeatability measurement. Reproducibility was not tested at this time. Because the method is faster (an important factor with unstable compounds) and contains few steps (consistent with accurate and precise results), the method is as rugged a procedure as can be devised.

Discussion

Accuracy

The question of accuracy was one of the principal reasons for the study, the answer to be found in either the measurement of an artifact in the CD and FID measurements or a loss of the vitamin in one of the steps in the usual purification scheme. From the chromatographic results, the only extraneous interference in the HCl and TCA extracts occurred equally in both the alkali blank and ferricyanide solutions, producing neither positive nor negative interference in either CD or FID measurements. The possibility that some component in the chicken extracts was causing greater conversion of thiamine to thiochrome in the extracts than in the standards was eliminated by the observation that the thiamine concentrations in the spikes were equivalent to the standards. The loss of thiamine in the standard method indicates that the rapid method yields a more accurate and higher value for the thiamine content of the chicken than the standard method does.

Table 5. Spike recovery (average percent of the standard)

	TCA/heat	HCl/heat	Enzyme digest	BR-70 effluent	Isobutanol extract
Av.	99.7	91.1	110.7	99.3	91.8
s	11.9	7.2	18.7	15.8	4.4

The loss of thiamine in the adsorption/elution step was observed by many authors (9, 25–27), and was listed by McRoberts (24) as a specific problem to be addressed in the determination of thiamine in enriched flour. Early in the history of the procedures for determining thiamine, Wang and Harris (28) listed as one of the special advantages of their procedure the elimination of the adsorption/elution step!

The problem is not just one of loss of thiamine in chicken on the Bio-Rex 70 columns, however. The concentration of thiamine in the chicken extracts was calculated by using the fluorescence of a standard that itself was adsorbed and eluted from the resin. That is, there was a loss of thiamine in the chicken extracts not observed in the standards. The problem was not one of incomplete elution. Pippen and Potter (25) found that a larger volume, 50 mL, was frequently required to effect total elution from Decalso. However, when we eluted Bio-Rex 70 with further portions of hot acid–KCl, we obtained no more thiamine. Furthermore, incomplete elution should have affected spike recovery, but no diminution in the spikes was observed (Table 5). The measured concentration of thiamine in a chicken extract appears to be lower simply because it is in the extract, which suggests that the thiamine in chicken differed from free thiamine. It apparently was not bound to any other compound, as its retention times on the Hypersil APS and DM-614 columns were the same as those of free thiamine. Oxidation of the chicken thiamine on the resin seems unlikely, as the oxidation would be expected to extend to the free thiamine in the spike. The nature of the problem of the poor elution of the thiamine in chicken extracts is not clear. Further investigation should yield interesting information on the state of thiamine in this meat.

Fluorescence

The fluorescent spectra of the various thiochrome derivatives and the molar fluorescences were dissimilar from some reports in the literature. Ishii et al. (10) reported excitation and emission spectra that show only 1 maximum in either spectra, but both of their peaks were skewed. Matsuda and Cooper (21) reported 2 emission maxima at 435–440 and 450 nm but only 1 excitation maximum at 365 nm. Mohamed et al. (29) reported spectra with a major excitation peak at 360 nm and a major emission peak at 425 nm, and minor peaks at 415 (excitation) and 360 (emission) nm. All of the thiochrome vitamers, both standards and from chicken, had the spectra shown here. The spectra were not of any compounds extracted from the plastic tubes used for the digestion, because the zero concentration thiamine samples showed no fluorescence. Ishii et al. (10) did not give details of how they measured the spectra, and

it may be that their instrument did not have the resolution of the Perkin-Elmer instrument we used, hence the skewing in their spectra.

Molar fluorescence values are also a problem. We did not observe any difference in the molar fluorescence of thiochrome and its phosphorylated vitamers, thiochrome monophosphate and thiochrome diphosphate. Ishii et al. (10) reported that the molar fluorescences of thiochrome monophosphate and thiochrome diphosphate were lower than that of thiochrome, in the ratio of 63:83:100, respectively. Conversely, Matsuda and Cooper (21) reported that the fluorescences of thiochrome diphosphate and thiochrome triphosphate had to be multiplied by factors of 0.87 and 0.80 because they were high in comparison with thiochrome. Lewin and Wei (30) did not find any difference in the molar fluorescences of the 3.

The problem is not simple. As Risinger and Pell (31) reported, thiamine is readily oxidized to the disulfide, and Ryan and Ingle (32) and Rose and France (33) observed multiple oxidation products during the formation of thiochrome. Furthermore, the reaction does not go completely to thiochrome (34). Barger et al. (35) reported that the reaction, as they ran it, resulted in only 30% conversion of thiamine to thiochrome. Marquez et al. (23), using a kinetic method to determine thiamine, found maximal production of thiochrome at about 10 min in their system, with a gradual decrease of fluorescence after that time. Under these conditions, it is possible that the vitamers might not be oxidized to the same extent depending on the conditions, but taking the literature *in toto*, and in view of our own results, we believe it is correct to assume that the molar fluorescences of the vitamers are all the same.

Precision

Precision values in the literature are limited to total procedures and a limited number of samples. A survey of the articles in which precision values are reported showed a range of 0.5–50% for the coefficients of variation, with an average of about 8%. Coefficients of variation reported for chicken thiamine are 4% for replicate determination (3) (in this study, ca 1.5%); 8.1–21% (17) and 8.6–28.6% (18) for replicate samples (in this study, 3.37%). The higher coefficients of variation in the last 2 may be due to the chicken-to-chicken variation, which was found to be 15.7% in this study. From these comparisons, the precision of the rapid method is better than reported in the literature. One of the major problems in precision comes at the fluorescence measurement step, where a very small amount of light scattering can result in very large deviations in the measured peak. Light scattering results in high and variable peaks; it was observed that the outliers were almost all in the direction of greater values. The use of a cut-off filter as close to the desired emission wavelength as possible reduces or eliminates variation from this source (Figure 3) and increases the precision of the measurements.

Conclusions

The determination of thiamine has to be a balance between elimination of interferences and losses introduced by excessive handling or too many steps in preparation. The results of this study indicate that the AOAC rapid method, **953.17** (1), when modified, yields more precise and accurate values for the thiamine in chicken than does the longer method, **942.23**, principally because of losses in the adsorption/elution and thiochrome formation/extraction steps of the latter. The use of trichloroacetic acid, flow injection determination, and cut-off filters as close to the emission wavelength as possible yields the best results.

References

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